Using microsatellite markers for genetic individualization of European wildcats (*Felis silvestris*) and domestic cats

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Abstract. Results of a pilot study are presented that aim to genetically individualize wildcats and domestic cats in blind proficiency tests. The genetic marker system employed is a suite of eight highly variable microsatellite loci specific for domestic cats that were cross-amplified in wildcats through gradient touchdown PCR. The resolution power of the detection system is discussed and future research directions are outlined.

Key words. Carnivora, *Felis silvestris*, Eifel region, microsatellites.

Introduction

The Eifel region in Nordrhein-Westfalen and Rheinland-Pfalz, Germany, is at the northwestern border of the Central European distribution area of the endangered European wildcat (*Felis silvestris*). Together with the adjacent regions of Belgium, Luxembourg, and France it has a fairly large population of wildcats, estimated at 800–1000 individuals. The population in our study area of about 2000 km² in the Eifel region of Nordrhein-Westfalen as the northern portion of this area is estimated at more than 200 animals. The "Artenschutzprojekt Wildkatze in Nordrhein-Westfalen (NRW)" was initiated to monitor this population and to develop guidelines for its conservation (Trinzen 1999, 2000).

Various factors like deforestation in general and fragmentation of the remaining forest areas, increasing number of human settlements, human intrusion into habitats suitable for wildcats as well as active prosecution had resulted in a drastic decline of the European wildcat in Germany and other countries of Central Europe until well into the second half of the 20th century. Only in a few regions small and scattered remnant populations were left when conservation measures started. The fact that most of these small populations are likely to contain hybrids between the European wildcat and feral individuals of the domestic cat and that such introgression is probably continuing has been a major concern in the conservation of the European wildcat because this poses a threat to the genetic integrity of the species. Such introgression is likely to have occurred at a small scale in the population studied probably since the domestic cat was brought to this part of Germany by the Romans about 2000 years ago.

The objective of the present study is to set up a first round of genetic tests to characterize and separate "pure" wildcats from morphologically similar feral domestic cats by DNA individualization profiles. The development of STR (short tandem repeats) or microsatellite loci in a range of domestic animals has created the potential for forensic identification of domestic animals (Dietrich et al. 1992, Ellegren et al. 1994). Microsatellites are a class of often highly polymorphic genetic

markers in eukaryotic genomes (Tautz 1989) that vary in the number of repeats of a simple DNA sequence stretch of 1 to 6 bases (different tandemly repeated numbers of these 'motif' variations are called 'allele sizes'). Exhaustive studies were published recently (Menotti-Raymond et al. 1999a,b, Murphy et al. 1999) reporting on the development of species-specific microsatellite markers for domestic cats, incorporated into a genetic recombination map of the cat. Examples of the promising use of this novel class of genetic tools are its employment in paternity analysis (Craighead et al. 1995), the analysis of introgressive hybridization (Dallas et al. 1995), and the forensic investigation of hairs (Menotti-Raymond et al. 1999a,b).

We report here on a pilot study employing PCR-based screening techniques to adopt a panel of eight feline microsatellite markers for the individualization of domestic cats and wildcats. We demonstrate the potential of this feline marker set for genotyping forensic tissue samples from road kills in a blind proficiency test.

Material and methods

Muscle tissue samples of 24 individuals (road kills and customs requisitions, Table 1) were collected and processed genetically in blind tests. Tissue samples were transferred to pure ethanol and kept there until processing. DNA was either extracted by means of the Nucleospin C+T kit (Macherey & Nagel, Düren, Germany), according to protocols supplied by the manufacturers, or by using a modified CTAB protocol (Gustincich et al. 1991).

Instrumentation

We describe in detail the genotyping protocols to be used in the experiments working with a temperature-gradient cycling machine and an automated DNA analysis system based on near infrared (IR) fluorescence technology using IR-labelled primers flanking microsatellite loci, which were amplified by touch-down PCR amplification.

A total of eight primer pairs designed for amplification in felines (Menotti-Raymond & O'Brien 1995) and by specific reactions in the domestic cat (Menotti-Raymond et al. 1999a,b) were screened for cross-species amplification in domestic cats and European wildcats. Primers were custom synthesized by MWG-Biotech (Germany), and the forward primers were end-labelled (5') with a IR800 infrared fluorescence label (Roy et al. 1996). We ran various gradient-touchdown programmes on the "T-Gradient" thermocycler (Whatman-Biometra) for quick detection of optimal amplification cycle profiles. PCR reactions (10 μl) were conducted in Mμlti™ Ultrastrips (Roth, Stuttgart) containing 0.26 mM dNTPs, 0.2 units of SigmaTaq (Sigma), the appropriate concentration of MgCl$_2$ and varying primer concentrations (see Table 2). KCl salt concentration of the 10XPCR buffer supplied with the enzyme was not varied. Approx. 50 ng genomic DNA gave proper amplification products. PCR programmes generally consisted of (i) an initial denaturation step at 94°C (3min); (ii) # of cycles of denaturation (1 min at 94°C), annealing (35 s) and extension (45 s at 72°C); (iii) 20 min at 72°C (# of cycles and exact annealing temperatures Ta for different primers are listed in Table 2).

Gel electrophoresis and visualization of PCR products was accomplished using a LI-COR Model 4000 automated DNA sequencer (LI-COR, Inc., purchased from MWG-Biotech). 25 cm gels of 0.25 mm thickness prepared by 6.5 % Long Ranger (Biozym) polyacrylamide matrices were run in 1xTBE electrophoresis buffer. Electrophoresis was controlled by the Quick SequencIR software that automates the process of focusing and autogaining the fluorescence signals from the gel, and setting the electrophoresis parameters (1500 V, 40 mA, 40 Watt, 50°C gel back plate heating, reading 7 image frames). Shark tooth combs for 64 wells were used for gel loading after the gel was mounted in the sequencer. We took full advantage of re-loading each gel up to four times while screening amplification success from series of PCRs. In the screening phase we simply used a scaled paper ruler with the 50-350bp STR Size

Table 1: List of tissue samples of cats genotyped in the blind proficiency tests.

Blind Probe Cat. No.	Collection site	Collection date	Identification
1, 2	Adenau, Rheinland-Pfalz, Germany	12. 08. 99	wildcat (w)
3	Wittlich, Rheinland-Pfalz, Germany	02. 02. 96	wildcat
4	Jünkerath, Rheinland-Pfalz, Germany	12. 09. 96	wildcat
5	Dahn, Rheinland-Pfalz, Germany	15. 11. 96	domestic cat (d)
6	Germany	07. 10. 96	domestic cat
7	Hüttersdorf, Rheinland-Pfalz, Germany	13. 10. 96	domestic cat
8	Stromberg, Baden-Württemberg, Germany	–	domestic cat
9	Udenbreth-Berk, Nordrhein-Westfalen, Germany	May 99	wildcat
10	Wittlich, Rheinland-Pfalz, Germany	14. 09. 99	wildcat
11	3 km W Belgian frontier, Belgium	08. 08. 99	wildcat
12	Antweiler, Euskirchen, Nordrhein-Westfalen, Germany	01. 04. 99	domestic cat
13	Germany	12. 12. 98	domestic cat
14	Nettersheim, Nordrhein-Westfalen, Germany	24. 09. 99	domestic cat
15	Bienwald, Rheinland-Pfalz, Germany	15. 09. 95	wildcat
16	Freisbach, Rheinland-Pfalz, Germany	21. 03. 95	wildcat
17	Bienwald, Rheinland-Pfalz, Germany	30. 01. 95	wildcat
18	Deuselbach, Rheinland-Pfalz, Germany	15. 10. 96	wildcat
19	motorway A 8, Pirmasens – Zweibrücken, Rheinland-Pfalz, Germany	25. 10. 96	domestic cat
20	Nideggen, Nordrhein-Westfalen, Germany	09. 08. 99	wildcat
21	Germany	19. 01. 99	wildcat
22	Germany	04. 03. 99	wildcat
23	Stadtkyll, Rheinland-Pfalz, Germany	23. 06. 99	domestic cat
24	Germany	15. 12. 98	domestic cat
25	Cochem, Rheinland-Pfalz, Germany	03. 09. 99	wildcat

Table 2: Description of the microsatellite loci, size ranges and numbers of alleles, and basic information on the conditions for their PCR amplifications

Locus	Allele sizes 1)	Allele sizes 2)	Sample size	No. of alleles 2)	Ta (°C)	(MgCl$_2$) mM	(primer) μM	PCR cycles	DNA conc. ng/μl	dilution factor for scoring
Fca096	184–224	182–229	24	11	55.8	1.5	0.5	27	50	1:30
Fca126	139–145	129–145	24	9	55.4	1.5	0.5	27	50	1:30
Fca132	137–153	137–162	24	9	52.6	1.5	0.5	27	50	1:30
Fca031	221–241	217–238	24	8	57.3	1.5	0.5	28	50	1:18
F115	193–217	187–242	24	20	54	1.5	0.5	25	50	1:30
Fca035	136–150	111–163	24	11	53.5	1.5	0.5	30	50	variable
Fca105	189–197	176–204	24	10	53	1.5	0.5	25	50	1:30
Fca124	110–134	115–130	24	8	51	1.5	0.5	27	50	1:30

1) listed from literature: Menotti-Raymond & O'Brien (1995), Menotti-Raymond et al. (1999,b)
2) this study

Standard (LI-COR supplied by MWG-Biotech) drawn on it to estimate fragment sizes. After identifying the optimal conditions of the PCR for each primer pair, we amplified the microsatellites for many individuals and again checked individual amplification profiles on the screen to be sure to have real microsatellite patterns. In most cases amplified products were serially diluted (dilution factors see Table 2) with loading buffer for optimal automated detection as estimated from the screen image. Loading of the samples was repeated, now running two STR size standard lanes alongside with 12 sample lanes. The whole set of twenty-four samples were processed in one electrophoresis run separating the fragments produced by a single primer pair. The progression and quality of the microsatellite amplicons can be visualized instantly on the screen of the computer during the separation time of 45 min to 120 min. Allelic patterns are then displayed as autoradiogram-like images, scored visually and quantitated by Gene ImagIR software.

Upon completion of the run the screen gel image was analyzed by computerized fragment analysis ('RFLPScan', ScanAnalytics, Billerica, MA), which comes also with databasing facilities for gel analyis. The RFLPscan system automates the analysis of DNA profiling digitized gel images (STR fragment patterns) and produces accurate quantitative results for determining fragment sizes. The database function provides a "matching bands" option and generates allele bin classes which include all the bands at a given matching position across all lanes of all gels analysed. Sizing precision has a variance of less than 0.5 percent standard deviation with only three 350bp standard DNA ladder lanes per gel.

Data analysis

As an introductory step multilocus genotypes of all 8 microsatellite loci were presence-/absence-coded for each specimen resulting in a total of 86 allelic states, which were used as variables in the calculations. Euclidean distances were calculated between the specimens analysed. The matrix was subjected to a multidimensional scaling procedure to reduce the variable space to few dimensions (SPSS for Windows v. 9). A plot of dimension 1 against 2 was used to visually demonstrate genetic differentiation between the individuals on a 2D plane. The final plot was labelled according to traditional identification (Table 1).

Observed and expected heterozygosities were analyzed using BIOSYS-1 (Swofford & Selander 1981). Average heterozygosities were calculated in two ways: (1) the proportion of individuals sampled that are actually heterozygous ("direct-count"), assigned to the relevant genepool on the basis of the outcome of the multidimensional scaling procedure, (2) the unbiased estimate, based on conditional expectations (Nei 1978) according to the Hardy-Weinberg proportion. When more than two alleles are present in a population sample, a pooling procedure as follows was applied and pooled genotype frequencies were used in a chi-square test with one degree of freedom: Three genotype classes (homozygotes for the most common allele, heterozygotes for the most common allele and one of the other alleles, and all other genotypes) were used for the calculation of the test value.

Genotypic frequencies at each polymorphic locus were further checked for differences between the two resulting genepools (wildcats and domestic cats) by a contingency table analysis of heterogeneity among population samples provided by BIOSYS-1. The test employs the familiar Pearson chi-square statistic for an M x N contingency table with (M–1) (N–1) degrees of freedom, where M is the number of populations and N the number of alleles.

We used the module FSTAT of BIOSYS-1 for analyzing genetic subdivision of population samples by F-statistics (Wright 1978) in the formulation defined by Weir and Cockerham (1984). The fixation index F_{ST} measures the amount of differentiation among subpopulations relative to the limiting amount under complete fixation. Finally, Cavalli-Sforza & Edwards (1967) geometric chord distance which performs well with microsatellite data was calculated for the two samples to depict genetic differences between domestic and wildcats. Multilocus allele frequencies were square-rooted and population samples lie on the surface of a hyperplane with radius one. The chord length of the two vectors connecting the sample coordinates on the surface was used as the genetic distance.

Results

Figure 1 shows the high quality of the genotypic profiling images detected at the eight cat microsatellite loci for the 24 specimens tested. Figure 2 is an example of the pictorial interpretation of the fragment pattern of the microsatellite locus Fca124 (upper part) from which the individual genotypes were derived for further calculations (lower part). The 2D plot (Fig. 3) clearly demonstrates the separation of domestic and wildcat genepools based on scaled distances between the composite genotypes at eight microsatellite loci of blind samples. The representation of the individual samples in the 2D-plot was labelled after the multidimensional scaling had been performed. The number of allele bin classes and respective ranges of fragment sizes are shown in Table 2. Allele frequencies at microsatellite loci calculated a posteriori from the pooled domestic cat and wildcat genepools are given in Table 3.

Overall significance testing by exact probabilities for pooled genotype class categories in both the "wildcat" and the "domestic" cat sample did not detect significant deviations from Hardy-Weinberg expectations though sample sizes were small, and samples were surely not taken from homogeneous populations. However, when observed and expected heterozygotes are compared on a single locus basis, there was a highly significant deficiency detected for both samples at the locus FCA096 (wildcats: p=0.0011; domestic cats: p=0.027). Contingency chi-square analysis at all loci in general (p < 0.001: all loci combined) showed significant differences in the allele frequency distributions between wildcats and domestic cats (p < 0.055: loci F115, Fca031, Fca035, Fca096, Fca126, Fca132), whereas the loci Fca105 and Fca124 showed no differences between the two samples.

Table 3: Allele frequencies (n = allele counts) in wildcats (w) and domestic cats (d).

|	Population				Population	
Locus	w	d	Locus	w	d	
F115						
allele	n = 12	n = 7				
187	0.000	0.071	209	0.125	0.071	
193	0.000	0.071	212	0.042	0.071	
195	0.042	0.000	213	0.042	0.000	
196	0.000	0.071	215	0.083	0.000	
197	0.000	0.214	221	0.042	0.000	
198	0.042	0.000	225	0.000	0.071	
202	0.125	0.000	230	0.083	0.071	
204	0.083	0.000	232	0.042	0.000	
205	0.167	0.000	237	0.000	0.143	
207	0.083	0.000	242	0.000	0.143	
Fca031						
allele	n = 12	n = 7				
217	0.000	0.143	233	0.042	0.071	
225	0.125	0.000	234	0.042	0.286	
227	0.000	0.071	237	0.042	0.143	
231	0.625	0.286	238	0.125	0.000	
Fca035						
allele	n = 12	n = 10				
111	0.000	0.000	150	0.167	0.000	
137	0.125	0.600	151	0.000	0.000	
139	0.208	0.000	153	0.042	0.000	
142	0.083	0.000	155	0.083	0.000	
144	0.167	0.200	163	0.083	0.000	
147	0.042	0.200				
Fca096						
allele	n = 14	n = 10				
183	0.000	0.150	219	0.500	0.050	
206	0.000	0.250	221	0.107	0.000	
208	0.000	0.400	224	0.071	0.000	
213	0.036	0.100	228	0.036	0.000	
215	0.107	0.050	229	0.036	0.000	
217	0.107	0.000				
Fca105						
allele	n = 13	n = 8				
176	0.000	0.063	195	0.192	0.125	
187	0.077	0.063	198	0.154	0.125	
188	0.038	0.000	200	0.077	0.000	
191	0.192	0.063	203	0.038	0.375	
194	0.154	0.063	204	0.077	0.125	
Fca124						
allele	n = 14	n = 9				
116	0.107	0.222	125	0.071	0.111	
118	0.071	0.000	127	0.429	0.222	
120	0.143	0.222	129	0.143	0.167	
121	0.036	0.000	130	0.000	0.056	
Fca126						
allele	n = 11	n = 8				
129	0.045	0.000	139	0.000	0.063	
131	0.182	0.000	142	0.045	0.125	
133	0.364	0.063	143	0.000	0.063	
135	0.364	0.125	145	0.000	0.250	
137	0.000	0.313				
Fca132						
allele	n = 13	n = 10				
137	0.000	0.150	154	0.269	0.300	
142	0.000	0.250	156	0.308	0.050	
145	0.038	0.100	158	0.115	0.100	
148	0.077	0.000	162	0.115	0.000	
152	0.077	0.050				
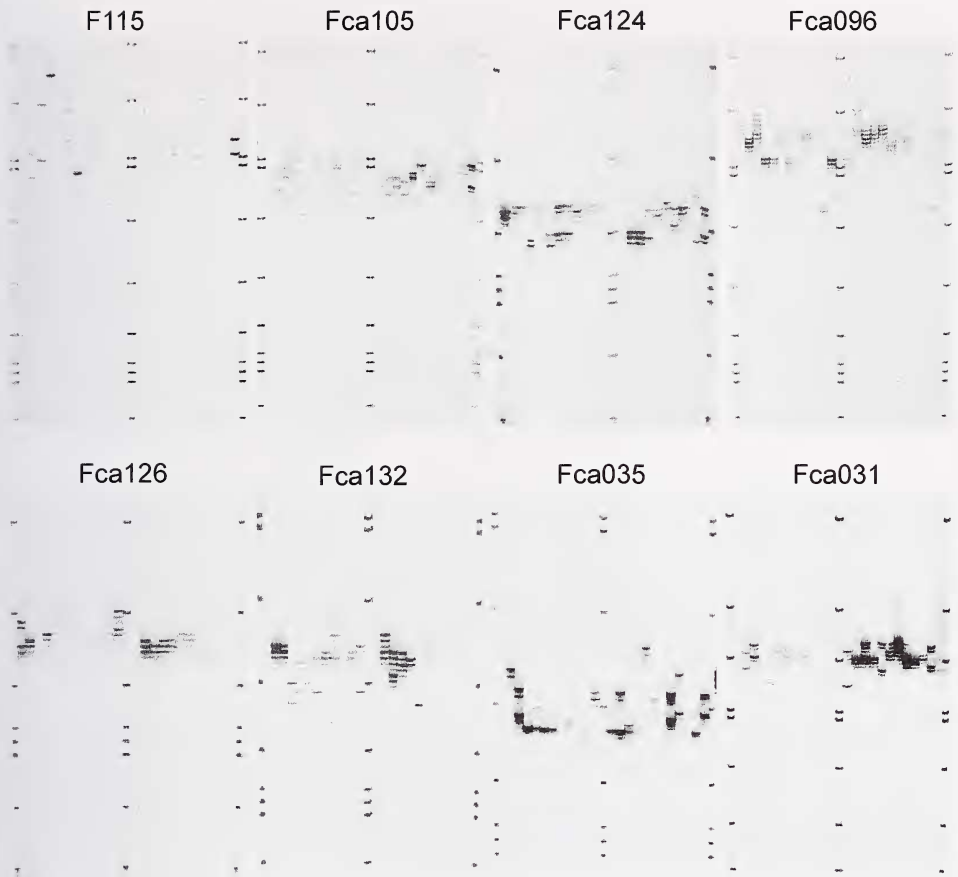
Fig. 1: Eight gel images of electrophoretic separation of microsatellite loci for 24 cats; for interpretation see Fig. 2.

Cavalli-Sforza & Edwards (1967) geometric chord distance was estimated to be 0.597 (to be compared with Nei's unbiased genetic distance [1978] of 0.600). Assuming a range of mutation rates between 10^{-4} and 10^{-5} per generation these genetic differences would accumulate over a time span of 300 to 3000 years (according to the formula of Nei 1978).

Discussion

Genetic systems such as species-specific microsatellites are comparatively easy to automate with highly optimized technical equipment, here the combination of a temperature-gradient thermocycler and an automated sequencing system. The LiCOR automated DNA sequencing system with its advanced fragment analysis software is shown to allow microsatellite analysis with high throughput. Therefore, it can be con-
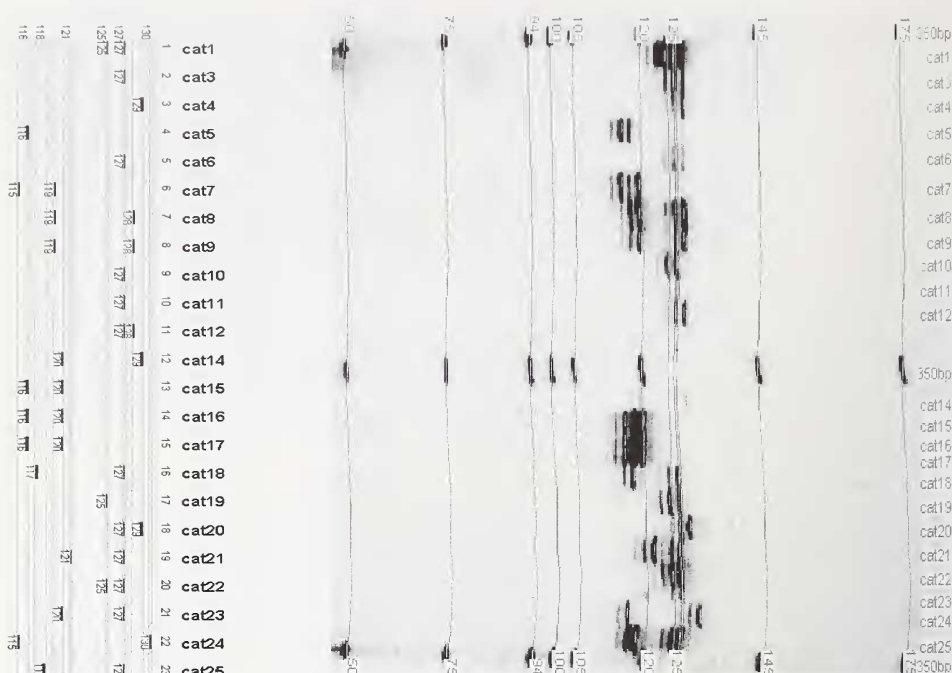
Fig. 2: Gel image of the microsatellite locus Fca124 and pictorial representation of the genotypes: For each of the 24 samples, PCR reactions of the Fca124 microsatellite locus were loaded into a single lane. 350bp molecular weight standard were run in the left, middle and right lane. Lanes were indicated with the samples IDs and numbers along the marker lanes give fragment sizes in base pairs.(upper part). Genotypic interpretation for each individual based on fragments' sizing in relation to the 350bp DNA standard. (lower part).

sidered the "state-of-the-art" method to easily produce the data necessary to assess the genetic architecture of cat populations.

We performed preliminary genetic tests using a panel of eight feline microsatellite markers in a blind proficiency test in order to individualize 24 cats. The results showed a high discrimination power of the method employed. The planar configuration consists of two clearly separated groups, one close cluster comprising domestic cats and a second one combining wildcats. Although sample size is certainly too low in this pilot study (15 presumed wildcats, 9 domestic cats) to establish a reliable database for allele frequencies of microsatellites for each of the groups, it is noteworthy that this first approach to the basic data fulfils the demands of an objective test requiring no a priori information as to species allocation of the samples. In this respect multidimensional scaling provides the potential of an "assignment test" (Paetkau et al. 1997).

Binning of alleles, i. e. a grouping of alleles that are likely to contain the same-sized microsatellite repeat (Table 2), in all 24 samples yielded an average number of 8.0 ± 0.9 alleles per locus for the wildcat and a mean number of alleles per locus of 6.6 ± 0.7 for domestic cats. Generally, microsatellite loci were highly polymorphic,

with a minimum of 8 alleles at the loci Fca031 and Fca124, and a maximum of 20 alleles for F115. Hence, genetic variability detected with microsatellites was much higher than the respective variation detected by means of allozymes (Randi & Ragni 1991).

Overall significance testing by exact probabilities for pooled genotype classes did not show deviations from the Hardy-Weinberg equilibrium at the loci scored. We interpret the overall conformity of genotype distributions to Hardy-Weinberg (panmictic) expectations as an indication that the study area was not large enough to have excessive internal genetic structure, which should be true both for wildcats and surrounding rural domestic cat populations. It must be kept in mind, however, that despite being unbiased, the probability estimates obtained by the test procedure generally deviate further from the exact probabilities than do those calculated in the usual manner with no pooling. The above also holds for multi-allelic loci. In this case the pooling necessary for the exact test may obscure real deviations from Hardy-Weinberg expectations. The exception can be demonstrated by inspection of the STR locus Fca096, which in both groups expressed too few heterozygotes. The segregation patterns deviated significantly from the Mendelian predictions due to the number of heterozygotes expected. A probable explanation is that non-amplification of certain alleles due to substitutions, insertions, or deletions within the priming sites led to apparent null-alleles. Another plausible explanation is that sometimes allele scoring is problematic, especially in dinucleotide microsatellite loci (Figs 1, 2), because Taq polymerase-generated slippage products (shadow bands) are often seen.
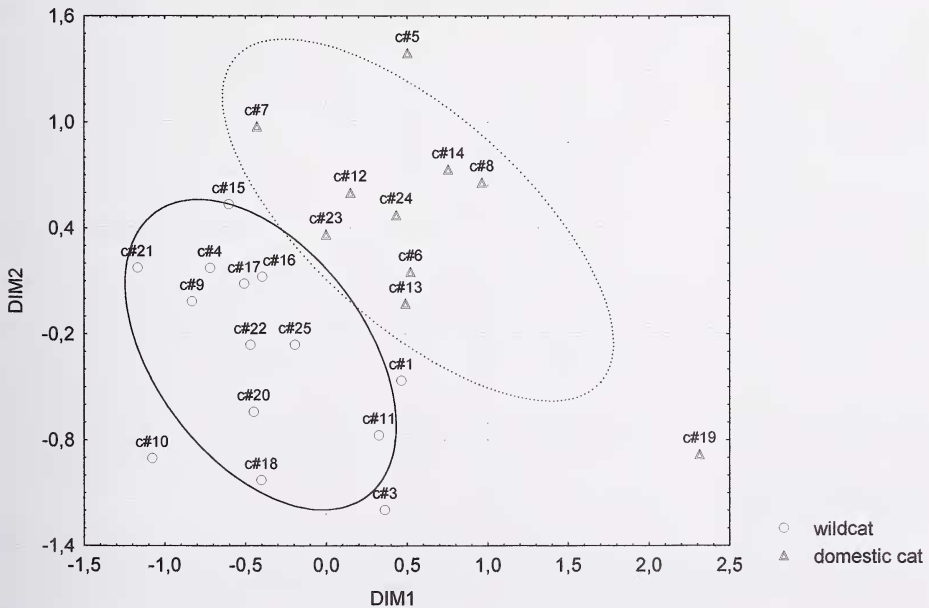

Fig. 3: Plot of dimension 1 against 2 of the multidimensionally scaled Euclidean distances between the 24 individuals based on their multilocus genotypes (see text).

Contingency chi-square analysis of allele frequency distributions at all loci demonstrated highly significant differences in the two genepools. The most important conclusion from these gaps in allele frequency distributions is that the two genepools probably are genetically isolated. This is in accordance with current hypotheses on cat domestication. The domestic cat belongs to the African wildcat lineage and the European and African wildcats diverged approximately 20000 years ago (Randi & Ragni 1991).

        Since genetic drift is the primary source driving genetic distances at the populational scale of small and isolated populations and the primary mode of mammalian microsatellite evolution is assumed to behave according to a stepwise-mutation-model (SSM; Amos 1999) we can use reasonable estimates of mutation rate $\mu \leq 0.0001$ per generation (t = 1 year) to estimate the time since divergence of the lineages. The mean value of either Cavalli-Sforza & Edwards geometric chord distance or Nei's unbiased genetic distance D correspond to a period of approximately 3000 years. Even conservative estimates of $\mu \leq 0.00001$ per generation yield an estimated time of 30000 years since divergence. Assuming constant population size and no gene transfer, D is expected to remain relatively linear under the SMM up to values of around 0.5 (Nei 1987), thus the divergence times may be overestimated. Our estimate of the minimum divergence time corresponds to the initial phase of domestication of the domestic cat from the Arabian wildcat believed to have occurred in Egypt 5000 years ago, with the resulting domestic cat spread mainly by the Romans 2000 years ago (Clutton-Brock 1987). The upper estimate dates back to the time frame of lineage sorting of European from African wildcats estimated by biochemical markers (Randi & Ragni 1991).

Results from F-statistics calculations describing the genetic differentiation of the two cat groups include the probability of open genepools, at least by demographic pressure through free-ranging domestic cats in wildcat habitats. The problem of potential hybridization was not addressed by this study, but an estimate of effective gene flow N_em of 2–3 individuals derived from the F_{ST}-value between the samples (0. 091 ± 0.025) indicated a relatively high probability of geneflow via present and historical incidental hybridization between wildcats and domestic cats (cf. Gaggiotti et al. 1999). To address the problem of interbreeding in the future one can calculate (using binomial sampling theory) the chance probability that two specimens have the same composite genotype taken from identical allele frequency distributions to be only 0.029 for the genepool of domestic cats and 0.083 for wildcats. Hence, on the assumption that allele frequency distributions which have been proven to be different between domestic cats and wildcats in our pilot study will diverge further by increased sampling, these estimates translate to values greater than 97.1 and 91.7 to find different composite cat genotypes with only 8 loci. Therefore, the method offers high potential to improve the individualization capacity to near security when screening more genetic markers in genetic tests.

Any study that involves sampling generates two sources of sampling error, biological and statistical. In this limited pilot study, we suggest that statistical error predominates because of small sample sizes and a limited number of genetic markers applied. Thus, all numerical estimates only give preliminary indications of possible "true" population parameters which have to be evaluated and confirmed on a broader data basis. On this basis we hope to be able to reassess whether there is a suite of

morphological characters to discriminate between European wildcats, domestic cats and their hybrids and to outline the degree of introgression between wildcats and domestic cats in the historic and the extant populations of the wildcats in the Eifel region.

Zusammenfassung

In dieser Pilotstudie untersuchten wir zur individuellen DNA-Typisierung von Haus- und Wildkatzen 8 neutrale nukleäre Genmarker, sogenannte Mikrosatelliten. Ohne vorherige Kenntnis von deren „Art"-Zugehörigkeit wurde aus Gewebeproben von 24 Tieren genomische DNA mit Hilfe der Polymerasekettenreaktion (PCR) locus-spezifisch vervielfältigt und anschließend mit einem automatischen Sequenzierer elektrophoretisiert und ausgewertet. Die vorliegende Studie konnte belegen, dass sich Haus- und Wildkatzen aufgrund des hohen Polymorphismus der Mikrosatelliten schon auf der Grundlage weniger genetischer Merkmalssysteme mit Hilfe exploratorischer statistischer Verfahren unterscheiden lassen.

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